Bayer HealthCare

Report No.: A53191

PES Vorstufe 2342

IN VITRO MICRONUCLEUS TEST WITH CHINESE HAMSTER V79 CELLS

Report of study TOXT4081014

BY

DR. A. SUTTER / S. JUNG

PERFORMING LABORATORY:

Bayer HealthCare AG
GDD-GED-TOX-ITOX Genetic Toxicology
13342 Berlin
Germany

SPONSOR:

Bayer MaterialScience AG
51368 Leverkusen
Germany

Study Completion Date: 07 Oct 2011

GLP Compliance Statement

Test Item : PES Vorstufe 2342
Study No. : TOXT4081014

The study was conducted in compliance with the OECD Principles of Good Laboratory Practice as revised in 1997 [ENV/MC/CHEM(98)17] and with the revised German Principles of Good Laboratory Practice according to Annex I German Chemicals Act (Bundesgesetzblatt, Volume 2008, Part I, No 28, 1173-1184, issued July 11, 2008).

Dr. A. Sutter
Study Director

6 October 2011
Date

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Quality Assurance Statement

Study No.: TOXT4081014

Test Item: PES Vorstufe 2342

On the dates given below inspections were conducted by the Quality Assurance to ensure that no deviations exist that are likely to affect the integrity of this study.
The Quality Assurance Unit monitors the conduct of each study by study-based inspections or by process-based inspections of a similar type of study if the short-term nature of a study precludes inspection while it is in progress.
Routine procedures and the equipment used in the relevant laboratory areas are inspected regularly and reports are made in accordance with our SOPs.

* (study plan amendments, if any, were duly audited and reportet to the Study Director and Management)

Date of Audits / Inspections	Phases Audited / Inspected		Date of Report to Study Director and Management
Mar-11-2011	Study Plan*		Mar-11-2011
Mar-16-2011	study based	Preparation / Processing of Samples, Labelling, Raw Data / Documentation	Mar-16-2011
Jul-12-2011	study based	Labelling, Raw Data / Documentation, Preparation of Formulation	Jul-12-2011
Oct-04-2011	Main Report	1.Draft	Oct-04-2011
Oct-06-2011	Main Report	Final Draft	Oct-06-2011

The results of this study including the methods used have been checked on the basis of the current SOPs.
They have been correctly reported and the report reflects the raw data.
In case of a multi-site study audits at the test sites are presented in the QA Statement of the Principal Investigator's report (see appendix).

Quality Assurance Unit
Global R&D Quality, GLP-Mgmt.

Date: 06 Oct 2011

Signature: _____
G. Münster

1. Signatures

Dr. A. Sutter
Study Director

07.10.2011
Date

Dr. T. Steger-Hartmann
Head of Investigational Toxicology
Test Facility Management

14.10.2011
Date

2. Summary

The purpose of this study was to investigate whether PES Vorstufe 2342 can induce chromosome breakage (structural chromosomal aberrations) or misdistribution of chromosomes leading to aneuploidy, both of which are measured by an increase of the frequency of micronuclei containing mammalian cells in the absence and presence of an extrinsic metabolizing system.

PES Vorstufe 2342 was examined for mutagenic activity in the micronucleus test in vitro. In the appropriate guideline for the in vitro micronucleus test (see 4.6) a second independent assay is requested if the first one was negative or produced equivocal results. In this study, two independent assays were performed, the first assay conducted with a treatment time of 4 hours (pulse treatment), consisting of one experiment in the absence and one experiment in the presence of an extrinsic metabolizing system (S9 mix). In the second assay, an experiment without S9 mix was performed with treatment time extended to 24 hours (continuous treatment).

As a rule the highest concentration tested should either be cytotoxic or correspond to the solubility limit of the test item (microscopically visible precipitates). Solid test items showing neither cytotoxicity nor precipitation should be tested up to 10^{-2} mol/L or 5 mg/mL (see 4.6, OECD guideline 487).

Negative controls (dimethyl sulfoxide, cell culture medium) and appropriate positive controls with known mutagens (mitomycin C, vinblastine sulfate, cyclophosphamide) demonstrated the suitability and sensitivity of the test system.

In both assays precipitates of the test item could be observed at 200 µg/mL.

Cytotoxicity was not observed in the first assay with pulse treatment (4 hours) in the absence or presence of S9 mix up to a precipitating concentration of 200 µg/mL. In contrast, 42 % cytotoxicity (determined as 100 % - relative increase in cell count, RICC) could be observed in the independent repeat experiment without S9 mix at a concentration of 200 µg/mL (24 hours treatment). Only a slight cytotoxicity evident from a 19 % decrease of the proliferation index (PI) was observed at this concentration.

The micronucleus test showed no increase in the frequencies of micronucleus containing V79 cells treated with PES Vorstufe 2342 in the absence (4 hours or 24 hours treatment) or in the presence of S9 mix.

Evaluation of the data does not indicate that PES Vorstufe 2342 is a mutagen in the micronucleus test in vitro, when tested up to a precipitating concentration of 200 µg/mL in the absence (4 hours treatment or 24 hours treatment) or in the presence of metabolic activation.

3. Introduction

When cytogenetic damages leading to chromosomal breakage (clastogenic effects) or misdistribution of chromosomes (aneugenic effects) are induced during mitosis, chromosomal fragments or a whole chromosome may be separated from the main nucleus. In interphase the separated fragment or chromosome can form a tiny nucleus (micronucleus) that exists independently of the main nucleus in the cytoplasm. Micronuclei can be seen in a wide variety of cell types. In the micronucleus test employed in the present study in vitro cultivated V79 cells were used. V79 cells were derived from fetal lung tissue of Chinese hamsters and are one of the cell lines most widely used for mutagenicity studies (3). In common with all cell lines they do not possess the full ability of mammals to activate promutagenic and procarcinogenic compounds. To overcome this deficiency the compounds are tested in the presence of an exogenous metabolizing system. Postmitochondrial supernatant fraction from liver of Aroclor 1254-treated male rats and a NADPH-generating system have been successfully used in prokaryotic and eukaryotic in vitro systems for the activation of various compounds.

Study Initiation Date	: 10 Mar 2011
Experimental Starting Date	: 15 Mar 2011
Study Start Date	: see Experimental Starting Date
Experimental Completion Date	: 11 Aug 2011

The study plan, raw data, specimens and the final report are retained in the archives specified by the test facility Nonclinical Drug Safety of Bayer HealthCare AG in Berlin.

4. Material and Methods

4.1. Substances

4.1.1. Test substance

Name of test substance	:	PES Vorstufe 2342
Batch number	:	LB06603520
Content	:	estimated 100 % (indicated by the sponsor)
Approved	:	until 22 Oct 2010 (Identity test on 19 May 2010) until 21 Mar 2011 (Check of expiry date, 11 Oct 2010) until 11 Sep 2011 (Check of expiry date, 23 Mar 2011)
Visual appearance[1]	:	colorless liquid
Storage	:	at 2°- 8°C, dark
Chemical name	:	Castor Oil, reaction product with Soybean Oil
Structure	:	not indicated by the sponsor
EC-No.	:	919-697-6
Indication	:	binder for coating material and adhesive

The batch used was analytically examined prior to study initiation and was approved for use for the test period. A stability test in the solvent[2] did not reveal significant degradation of the active ingredient.

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[1] does not reflect chemical composition
[2] solvent will be used as technical term, even if the formulation is a suspension or a emulsion

4.1.2. Positive Controls

Mitomycin C (batch no. 049K0788, Sigma Aldrich) and vinblastine sulfate (batch no. BCBC2023, Sigma Aldrich) were used as the positive controls in the experiments without metabolic activation. Cyclophosphamide (batch no. 8L534C, Baxter Oncology GmbH) was used as positive control in the experiments with S9 mix.

4.2. Study Design

The study was performed according to the current version of SOP TX. ME 793 which complies with the internationally accepted guideline (see 4.6), and taking into consideration the references (1), (2) and (3) where appropriate.

4.2.1. Culturing of V79 Cells

Stocks of the V79 cell line (supplied by Prof. Miltenburger, Darmstadt, Germany) are stored in liquid nitrogen allowing the repeated use of the same culture batch. Consequently, the parameters of the experiments remain similar because of the reproducible characteristics of the cells. The cells have a stable karyotype with a modal chromosome number of 22. Cells were routinely checked for mycoplasma contamination and karyotype stability.
Thawed stock cultures were propagated at 37 °C and 5 % CO_2 in 25 cm^2 plastic flasks. Seeding was performed with about 1 x 10^5 cells per flask in 5 mL MEM with Earl's Salts and Glutamax I (Minimal Essential Medium, Life Technologies, Eggenstein, Germany) supplemented with 10 % fetal bovine serum (FCS) and 1 % Penicillin/Streptomycin-solution (both from Life Technologies, Eggenstein, Germany). The cells were subcultured twice weekly after trypsination of the adherently growing cells.

4.2.2. Composition of S9 Mix

Liver homogenates (S9: 9000 x g fraction) were isolated in house from the livers of Aroclor 1254-induced male Sprague-Dawley rats. The used S9 fraction were derived from preparations dated 17 Nov 2008, color code black (protein content 27.2 mg/mL) or dated 01 Feb 2010, color code blue (protein content 26.6 mg/mL). Before treatment an appropriate quantity of S9 was thawed and mixed with co-factor solution to result in a final protein concentration of 2 % S9 in cultures.

4.2.3. Solvent Selection

DMSO (dried with a molecular sieve, 0.3 nm) was selected as solvent for PES Vorstufe 2342.
The stock solutions and further dilutions were prepared immediately before addition to the cell culture; thus no remarkable instability is expected which could influence the outcome of the study.

4.2.4. Treatment Protocol

The study was performed according to the pertinent SOP (see 4.2), and taking into consideration the references (1), (2), and (3) where appropriate.
In the initial assay a pulse treatment was performed, both without and with metabolic activation. The independent repeat experiment was performed without S9 mix as continuous treatment for 24 hours.
Exponentially growing cell cultures were trypsinized. About 5 x 10^4 cells per concentration were seeded in duplicate in 5 mL medium per Quadriperm-well (contains one slide). Cells were allowed to adhere for ca. 4 hours at 37 °C in a humidified atmosphere with ca. 5 % CO_2.
In the initial assay the medium was then replaced by medium containing the test substance (and 50 µL/mL S9 mix for the experiment with metabolic activation). After a 4-hour treatment period the cells were washed twice and incubated for further 20 hours in culture medium without the test substance.
In the second assay, an experiment without metabolic activation was performed, with an extended treatment period of 24 hours.
Thereafter, the adherently growing cells were exposed in situ to 0.4 % KCl hypotonic solution and fixed in glacial acetic acid/ethanol (1+3). The air-dried slides were stained with May-Grünwald and Giemsa solutions.

4.2.5. Determination of Cytotoxicity

Cytotoxic effects of the test item were assessed using the relative increase in cell count (RICC) as well as the proliferation index (PI) in the presence and absence of S9 mix. The results of the solvent controls were set 100 % and compared to the cultures treated with the test item.
For determination of the PI, 2000 cell colonies/cells per concentration (1000 per slide) were scored and the number of cells per colony calculated.
For determination of the RICC, about 5 x 10^4 cells per concentration were treated in duplicate in 24-well plates with the test item in the absence (pulse or continuous treatment) or presence (pulse treatment) of S9 mix. Thereafter cells were trypsinized

and resuspended in cell culture medium in a final volume of 1.5 mL. The cells were counted at the time of harvest using a CASY cell counter. RICC values were calculated from these numbers.

4.3. Test Concentrations and Selection Criteria for Reading

The highest concentration chosen for evaluation should be clearly cytotoxic, i.e. it should cause a cytotoxicity (determined by 100 % - RICC or PI) of approximately 55±5 %. Non-toxic compounds will be tested up to 10^{-2} mol/L but not higher than 5 mg/mL unless limited by solubility.
The concentrations of the test item, the controls and treatment durations used are compiled in TT 1. Based on the presence of microscopically visible precipitates at 200 µg/mL, three concentrations were selected for the reading of slides (in bold).

TT 1: Experimental design

Experiment No.	Test substance	Concentration (µg/mL)	S9 (4 hours)	Treatment duration
1	Medium control DMSO PES Vorstufe 2342 Mitomycin C Vinblastine sulfate	-- **1** % (v/v) 5,10, 25, **50**, **100** and **200** **0.1** 0.075	No	4 h (pulse treatment)
	Medium control DMSO PES Vorstufe 2342 Cyclophosphamide	-- **1** % (v/v) 5,10, 25, **50**, **100** and **200** 1 and **2**	Yes	4 h (pulse treatment)
2	Medium control DMSO PES Vorstufe 2342 Mitomycin C Vinblastine sulfate	-- **1** % (v/v) 5,10, 25, **50**, **100** and **200** 0.05 **0.002**, 0,003 and 0.004	No	24 h (continuous treatment)

The concentrations which were evaluated for micronuclei are printed in bold

4.4. Parameters

For the evaluation of the frequency of micronucleus containing cells which represents the genetic endpoint in this study, 2000 cells (1000 cells per slide) per concentration were scored. Only cells which divided at least once and, therefore, formed colonies of ≥ 2 cells were evaluated.

4.5. Assessment Criteria

So far no satisfactory mathematical methods are available for statistical analysis of mammalian cell mutagenicity experiments such as those performed here. Our experience has shown that the following predetermined descriptive criteria are the most useful for interpretation of the results.

The evaluation of the results is performed as follows:

- The test item is classified as mutagenic if one of the test substance concentrations induces a micronucleus frequency that is three times higher than the micronucleus frequency of the concurrent solvent control.
- The test item is classified as mutagenic if there is a reproducible concentration-related increase in the micronucleus frequency. Such an evaluation may be considered independently of the enhancement factor for induced micronucleus frequencies.
- In the evaluation of the test results historical control data obtained in the laboratory and scientific plausibility is taken into consideration.
- Any positive test result should be evaluated for its biological relevance.

4.6. Study Guidelines

The study was performed according to the following guideline:

OECD Guideline for the testing of chemicals 487
In vitro mammalian cell micronucleus test
OECD (2010)

4.7. Study Identification and Responsibilities

4.7.1. Type of Test and Study Number

Micronucleus test in vitro: TOXT4081014

4.7.2. Responsibilities

Study Director	: Dr. A. Sutter
Head of Genetic Toxicology	: Dr. T. Steger-Hartmann
Technicians	: D. Borkowsky, U. Gramlich, S. Jung, T. Naumann
Head of Nonclinical Drug Safety	: Dr. F. W. Jekat
Head of Investigational Toxicology	: Dr. T. Steger Hartmann

5. Results

The results of the solvent control confirmed the spontaneous micronucleus frequency which is characteristic for V79 cells.

Appropriate positive control items (mitomycin C, vinblastine sulfate, cyclophosphamide) showed the expected increase in micronucleus frequencies which demonstrates the ability and the sensitivity of the test system to detect cytogenetic damage.

Precipitates could be observed microscopically at a concentration of 200 µg/mL.

Cytotoxicity was not observed in the first assay with pulse treatment (4 hours) in the absence or presence of S9 mix up to a precipitating concentration of 200 µg/mL. In contrast, 42 % cytotoxicity (determined as 100 % - relative increase in cell count, RICC) could be observed in the independent repeat experiment without S9 mix at a concentration of 200 µg/mL (24 hours treatment). Only a slight cytotoxicity evident from a 19 % decrease of the proliferation index (PI) was observed at this concentration.

Thus, in the absence of limiting cytotoxicity, a precipitating concentration of 200 µg/mL was selected as the highest test concentration scored for micronuclei in all experiments.

The micronucleus test showed no increase in the frequency of micronucleus containing V79 cells treated with PES Vorstufe 2342 in the absence (both pulse and continuous treatment) or in the presence of S9 mix (pulse treatment) up to precipitating concentrations.

6. Conclusion

In conclusion, it can be stated that under the experimental conditions reported PES Vorstufe 2342 did not induce chromosome breakage (structural chromosomal aberrations) or misdistribution of chromosomes leading to micronucleus formation in V79 cells in vitro either in the absence or presence of metabolic activation.

7. References

1. Fenech M.
The in vitro micronucleus technique.
Mutat Res 2000; 455: 81-95

2. Kirsch-Volders M, Sofuni T et al.
Report from the In Vitro Micronucleus Assay Working Group.
Environ Mol Mutagen 2000; 35: 167-172.

3. Von der Hude W, Kalweit S et al.
In vitro micronucleus assay with Chinese hamster V79 cells - results of a collaborative study with in situ exposure to 26 chemical substances.
Mutat Res 2000; 468: 137-163

8. Historical Controls

8.1. Studies without metabolic activation

Summary of Historical Negative and Positive Controls
Mammalian cell line V79 (May 2002 – December 2009)

Medium control

Experimental part	Number of experiments	Range % micronucleus containing cells	Mean ± standard deviation
Continuous treatment 24 hours	45	0.1 – 1.8	0.8 ± 0.4
Pulse treatment 3 – 4 hours exposure with recovery of 20 – 21 hours	6	0.5 – 1.6	0.9 ± 0.4

Solvent control

Experimental part	Number of experiments	Range % micronucleus containing cells	Mean ± standard deviation
Continuous treatment 24 hours	44	0.3 – 2.3	1.1 ± 0.5
Pulse treatment 3 – 4 hours exposure with recovery of 20 – 21 hours	7	0.6 – 1.3	0.9 ± 0.3

Positive control mitomycin C 0.05 µg/mL

Experimental part	Number of experiments	Range % micronucleus containing cells	Mean ± standard deviation
Continuous treatment 24 hours	47	2.5 – 39.7	18.9 ± 8.3
Pulse treatment 3 – 4 hours exposure with recovery of 20 – 21 hours	5	3.8 – 15.0	8.1 ± 4.9

8.2. Studies with metabolic activation

Summary of Historical Negative and Positive Controls
Mammalian cell line V79 (May 2002 – December 2009)

Medium control

Experimental part	Number of experiments	Range % micronucleus containing cells	Mean ± standard deviation
Pulse treatment 3 – 4 hours exposure with recovery of 20 – 21 hours	47	0.2 – 2.2	0.9 ± 0.4

Solvent control

Experimental part	Number of experiments	Range % micronucleus containing cells	Mean ± standard deviation
Pulse treatment 3 – 4 hours exposure with recovery of 20 – 21 hours	48	0.3 – 2.0	1.0 ± 0.4

Positive control cyclophosphamide 2 µg/mL

Experimental part	Number of experiments	Range % micronucleus containing cells	Mean ± standard deviation
Pulse treatment 3 – 4 hours exposure with recovery of 20 – 21 hours	48	5.5 – 38.6	20.5 ± 7.2

9. Stability in Solvent

Results of the analyses for stability of
PES Vorstufe 2342

nominal value in mg/mL	content as % of start value after storage time in hours	
	0	24
1	100	83
200	100	85

According to these results PES Vorstufe 2342 is stable in the solvent at room temperature at concentrations ranging from 1 mg/mL to 200 mg/mL for at least twenty-four hours, a time interval which covers the time range from the preparation of the formulation to last treatment.

10. Tables 10.1 – 10.10

10.1. Relative increase in cell count (RICC), -S9, pulse treatment

Test substance	Concentration (µg/mL)	Cell number (mean of two wells)	RICC (%)[1)]	100 - RICC (%)
Medium	--	189150	100	0
DMSO (solvent control)	1 % (v/v)	189015	100	0
PES Vorstufe 2342	50	171975	88	12
	100	156825	77	23
	200 P	159900	79	21
Mitomycin C (positive control)	0.1	131745	59	41

P Precipitates

$$^{1)}\text{RICC (\%)} = \frac{\text{Cell number}_{\text{test substance}} - 50000}{\text{Cell number}_{\text{concurrent control}} - 50000} \times 100$$

10.2. Proliferation index, -S9, pulse treatment

Test substance	Concentration (µg/mL)	Cells/ cell colonies scored (n)	Colony size				Proliferation index (PI)		Cytotoxicity[1)] (mean, %)
			C1	C2	C4	C8	individual	mean	
Medium control	--	1000	162	728	103	7	1.96		
		1000	135	751	112	2	1.98	1.97	0
DMSO (solvent control)	1 % (v/v)	1000	217	726	56	1	1.84		
		1000	149	753	96	2	1.95	1.90	0
PES Vorstufe 2342	5	--[2)]							
	10	--[2)]							
	25	--[2)]							
	50	1000	196	700	102	2	1.91		
		1000	162	734	101	3	1.95	1.93	-3
	100	1000	185	714	98	3	1.92		
		1000	240	671	85	4	1.85	1.89	1
	200 P	1000	338	585	75	2	1.74		
		1000	308	584	106	2	1.80	1.77	14
Mitomycin C (positive control)	0.1	1000	302	632	65	1	1.77		
		1000	346	598	55	1	1.71	1.74	24

P Precipitates

$$PI = \frac{(1 \times C1) + (2 \times C2) + (3 \times C4) + (4 \times C8)}{\text{Number of cells/cell colonies scored}}$$

$$^{1)}\text{Cytotoxicity (\%)} = 100 - \frac{(PI_{\text{test substance}} - 1)}{(PI_{\text{corresp. solvent control}} - 1)} \times 100$$

[2)] = not scored

10.3. Micronucleus containing V79 cells, -S9, pulse treatment

Test substance	Concentration (µg/mL)	per 1000 cell colonies		%		Mean (%)	
		MN	≥ 6 MN	MN	≥ 6 MN	MN	≥ 6 MN
Medium control	--	3 10	0 0	0.3 1.0	0 0	0.7	0
DMSO (solvent control)	1 % (v/v)	6 7	0 0	0.6 0.7	0 0	0.7	0
PES Vorstufe 2342	5	--[1)]	--[1)]				
	10	--[1)]	--[1)]				
	25	--[1)]	--[1)]				
	50	13 4	0 0	1.3 0.4	0 0	0.9	0
	100	6 8	0 0	0.6 0.8	0 0	0.7	0
	200 P	9 9	0 0	0.9 0.9	0 0	0.9	0
Mitomycin C (positive control)	0.1	206 193	0 0	20.6 19.3	0 0	20.0	0

P Precipitates

MN = micronucleus containing cells

≥ 6 MN = cells containing 6 or more micronuclei

1) = not scored

10.4. Relative increase in cell count (RICC), +S9, pulse treatment

Test substance	Concentration (µg/mL)	Cell number (mean of two wells)	RICC (%)[1)]	100 - RICC (%)
Medium	--	141240	100	0
DMSO (solvent control)	1 % (v/v)	136193	100	0
PES Vorstufe 2342	50	145868	111	-11
	100	141248	106	-6
	200 P	124470	86	14
Cyclophosphamide (positive control)	2	123263	80	20

P Precipitates

$$^{1)}\text{RICC (\%)} = \frac{\text{Cell number}_{\text{test substance}} - 50000}{\text{Cell number}_{\text{concurrent control}} - 50000} \times 100$$

10.5. Proliferation index, +S9, pulse treatment

Test substance	Concentration (µg/mL)	Cells/ cell colonies scored (n)	Colony size				Proliferation index (PI)		Cytotoxicity[1] (mean, %)
			C1	C2	C4	C8	individual	mean	
Medium control	--	1000	176	713	110	1	1.94		
		1000	272	643	81	4	1.82	1.88	0
DMSO (solvent control)	1 % (v/v)	1000	224	705	69	2	1.85		
		1000	215	706	74	5	1.87	1.86	0
PES Vorstufe 2342	5	--[2]							
	10	--[2]							
	25	--[2]							
	50	1000	180	745	71	4	1.90		
		1000	223	703	72	2	1.85	1.88	-2
	100	1000	246	685	65	4	1.83		
		1000	299	617	82	2	1.79	1.81	6
	200 P	1000	265	674	55	6	1.80		
		1000	262	671	64	3	1.81	1.81	6
Cyclophosphamide (positive control)	2	1000	450	525	24	1	1.58		
		1000	466	488	46	0	1.58	1.58	34
	1	--[2]							

P Precipitates

$$PI = \frac{(1 \times C1) + (2 \times C2) + (3 \times C4) + (4 \times C8)}{\text{Number of cells/cell colonies scored}}$$

$$^{1)}\text{Cytotoxicity (\%)} = 100 - \frac{(PI_{\text{test substance}} - 1)}{(PI_{\text{corresp. solvent control}} - 1)} \times 100$$

[2] = not scored

10.6. Micronucleus containing V79 cells, +S9, pulse treatment

Test substance	Concentration (µg/mL)	per 1000 cell colonies		%		Mean (%)	
		MN	≥ 6 MN	MN	≥ 6 MN	MN	≥ 6 MN
Medium control	–	9 5	0 0	0.9 0.5	0 0	0.7	0
DMSO (solvent control)	1 % (v/v)	9 12	0 0	0.9 1.2	0 0	1.1	0
PES Vorstufe 2342	5	--[1)]	--[1)]				
	10	--[1)]	--[1)]				
	25	--[1)]	--[1)]				
	50	12 7	0 0	1.2 0.7	0 0	1.0	0
	100	15 7	0 0	1.5 0.7	0 0	1.1	0
	200 P	7 10	0 0	0.7 1.0	0 0	0.9	0
Cyclophosphamide (positive control)	2.0	188 227	0 2	18.8 22.7	0 0.2	20.8	0.1
	1.0	--[1)]	--[1)]				

P Precipitates

MN = micronucleus containing cells

≥ 6 MN = cells containing 6 or more micronuclei

[1)] = not scored

10.7. Relative increase in cell count (RICC), -S9, continuous treatment

Test substance	Concentration (µg/mL)	Cell number (mean of two wells)	RICC (%)[1)]	100 - RICC (%)
Medium	--	208950	100	0
DMSO (solvent control)	1 % (v/v)	158250	100	0
PES Vorstufe 2342	5	146093	89	11
	10	143438	86	14
	25	155025	97	3
	50	143258	86	14
	100	132750	76	24
	200 P	112545	58	42
Mitomycin C (positive control)	0.05	124673	47	53
Vinblastine (positive control)	0.002	117195	62	38
	0.003	92745	39	61
	0.004	110730	56	44

P Precipitates

[1)] RICC (%) $= \dfrac{\text{Cell number}_{\text{test substance}} - 50000}{\text{Cell number}_{\text{concurrent control}} - 50000} \times 100$

[2)] = not scored

10.8. Proliferation index, -S9, continuous treatment

Test substance	Concentration (µg/mL)	Cells/ cell colonies scored (n)	Colony size				Proliferation index (PI)		Cytotoxicity[1] (mean, %)
			C1	C2	C4	C8	individual	mean	
Medium control	--	--[2]							
DMSO (solvent control)	1 % (v/v)	1000	118	657	187	38	2.15		
		1000	129	637	196	38	2.14	2.15	0
PES Vorstufe 2342	25	--[2]							
	50	1000	130	635	202	33	2.14		
		1000	120	661	189	30	2.13	2.14	1
	100	1000	168	667	134	31	2.03		
		1000	137	687	149	27	2.07	2.05	9
	200 P	1000	191	687	103	19	1.95		
		1000	222	658	104	16	1.91	1.93	19
Mitomycin C (positive control)	0.05	--[2]							
Vinblastine sulfate (positive control)	0.002	1000	342	528	114	16	1.80		
		1000	333	530	129	8	1.81	1.81	30
	0.003	--[2]							

P Precipitates

$$PI = \frac{(1 \times C1) + (2 \times C2) + (3 \times C4) + (4 \times C8)}{\text{Number of cells/cell colonies scored}}$$

[1] $$\text{Cytotoxicity (\%)} = 100 - \frac{(PI_{\text{test substance}} - 1)}{(PI_{\text{corresp. solvent control}} - 1)} \times 100$$

[2] = not scored

10.9. Micronucleus containing V79 cells, -S9, continuous treatment

Test substance	Concentration (µg/mL)	per 1000 cell colonies		%		Mean (%)	
		MN	≥ 6 MN	MN	≥ 6 MN	MN	≥ 6 MN
Medium control	--	--[1)]	--[1)]				
DMSO (solvent control)	1 % (v/v)	10 15	0 0	1.0 1.5	0 0	1.3	0
PES Vorstufe 2342	25	--[1)]	--[1)]				
	50	5 7	1 0	0.5 0.7	0.1 0	0.6	0.1
	100	13	0	1.3	0		
		11	0	1.1	0	1.2	0
	200 P	11 17	1 0	1.1 1.7	0.1 0	1.4	0.1
Mitomycin C (positive control)	0.05	--[1)]	--[1)]				
Vinblastine sulfate (positive control)	0.002	220 246	3 2	22.0 24.6	0.3 0.2	23.3	0.3
	0.003	--[1)]	--[1)]				

P Precipitates

MN = micronucleus containing cells

≥ 6 MN = cells containing 6 or more micronuclei

1) = not scored

10.10. Summary of the results (pulse treatment –S9; continuous treatment -S9 and pulse treatment +S9)

Test substance	Concentration (µg/mL)	Cytotoxicity / 100-RICC (mean, %)			Cytotoxicity (PI) (mean, %)			MN (mean, %)			≥ 6 MN (mean, %)		
		-S9		+S9	-S9		+S9	-S9		+S9	-S9		+S9
		pulse	cont.		pulse	cont.		pulse	cont.		pulse	cont.	
Medium control	--	0	0	0	0	1)	0	0.7	1)	0.7	0	1)	0
DMSO (solvent control)	1 % (v/v)	0	0	0	0	0	0	0.7	1.3	1.1	0	0	0
PES Vorstufe 2342	5	nt	11	nt	1)	nt	1)	1)	nt	1)	1)	nt	1)
	10	nt	14	nt	1)	nt	1)	1)	nt	1)	1)	nt	1)
	25	nt	3	nt	1)	1)	1)	1)	1)	1)	1)	1)	1)
	50	12	14	-11	-3	1	-2	0.9	0.6	1.0	0	0.1	0
	100	23	24	-6	1	9	6	0.7	1.2	1.1	0	0	0
	200 P	21	42	14	14	19	6	0.9	1.4	0.9	0	0.1	0
Mitomycin C (positive control)	0.05	nt	53	nt	nt	1)	nt	nt	1)	nt	nt	1)	nt
	0.1	41	nt	nt	24	nt	nt	20.0	nt	nt	0	nt	nt
Vinblastine sulfate (positive control)	0.002	nt	38	nt	nt	30	nt	nt	23.3	nt	nt	0.3	nt
Cyclophosphamide (positive control)	2.0	nt	nt	20	nt	nt	34	nt	nt	20.8	nt	nt	0.1

P Precipitates

MN = micronucleus containing cells

≥ 6 MN = cells containing 6 or more micronuclei

nt = not tested

1) = not scored